Application and evaluation of male-specific bacteriophage as a process integrity or faecal contamination indicator in a pork slaughterhouse environment

A.J. MILLER, B.S. EBLEN, A. OSER AND W. BURKHARDT III. 1998. A male-specific bacteriophage plaque assay was evaluated as a faecal contamination or process integrity indicator for aspects of the pork slaughter process. Over 400 samples were tested including: sponge swabs from animal hauling trailer floors and dressed carcass surfaces; faecal material; water from slaughter sites; and water from each stage of wastewater treatment. Bacteriophage were observed in wastewater, trailers, slaughter process water and swine faeces. No bacteriophage were observed on dressed carcasses. Numbers of phage plaque-forming units per gram or millilitre showed greater variation and were usually lower than standard indicators, including total coliform or *Escherichia coli* counts. Among the applications studied, male-specific bacteriophage appear to be best suited for process control verification for wastewater treatment.

INTRODUCTION

For nearly a century, microbiologists have used non-pathogenic bacteria found in human and animal faeces to identify contaminated water and food, yet today, concern about the adequacy of these traditional systems to detect emerging or re-emerging disease threats has sparked a search for alternatives. To this end, male-specific RNA bacteriophage have received recent attention (Havelaar 1993; Araujo *et al.* 1997). Within this virus class, coliphage is a 21–30 nm diameter single-stranded RNA virus with a simple protein capsid which infects and replicates only within its host by attachment to the F^+ or sex pili. As coliphage prevalence is normally related to the presence of its *Escherichia coli* host, a common faecal bacteria, the virus may serve as an alternative indicator of faecal contamination in water and food.

Coliphage offers certain advantages as an indicator microorganism. First, the virus is reported to occur in high density in sewage and wastewater (Havelaar *et al.* 1984). In addition, it is similar to enteric viruses in chlorine and heat resistance (Havelaar and Nieuwstad 1985). This is an advantage over conventional indicators, as coliphage may serve as a more rigorous biomarker for the safety of treated water, or foods contacted by such water (Havelaar and Hogeboom 1983). Finally, the assay for these phages is rapid, economical, and simple to conduct.

The key rationale for coliphage use as an indicator microorganism is its inability to replicate without the presence of a faecal-associated *E. coli* host that contains F^+ pili (Burkhardt *et al.* 1992). Pili form only when the bacteria grow in the range of 30–45 °C (Seeley and Primrose 1982). As phage replication is dependent upon the presence of pili, there is little chance of encountering phage produced outside animals.

Bacteriophage was also suggested as a process integrity indicator for the removal of enteric viruses from shellfish (Doré and Lees 1995), for preparation of drinking water (Payment *et al.* 1985), and for water reclamation (Rose *et al.* 1996). These findings may be of value for certain segments of the animal slaughter industry, particularly those that are water intensive, such as chicken and swine processing. In fact, coliphage was observed in the faeces of pigs and chickens,

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which can yield F^+RNA phage in 100% of samples in densities up to 7 log plaque-forming units (pfu) g^{-1}. Moreover, male-specific bacteriophage was observed in the wastewater of swine and chicken slaughterhouses, with densities of 3 log pfu ml^{-1} (Havelaar *et al.* 1990).

As it has been associated with swine, it was hypothesized that male-specific bacteriophage could potentially serve as an alternative process integrity or faecal contamination indicator. Therefore, the objectives of this research were: (1) to investigate the ecology of male-specific bacteriophage in various pork processing operations; (2) to evaluate its use as a process integrity or faecal contamination indicator; and (3) to compare coliphage against more traditional bacteriological indicators in this environment.

MATERIALS AND METHODS

Bacteriophage strain and preparation

A strain of HS (F^+AMP) RR *Escherichia coli* was used. This strain contains a plasmid-bound ampicillin (AMP) resistance gene as well as chromosomal mediated genes for streptomycin and T_2 and T_4 phage resistance (RR). Isolated colonies were maintained at 4 °C on tryptone agar dishes: 10 g tryptone (Difco), 1 g dextrose (Difco), 5 g NaCl (Mallinkrodt, Paris, KY, USA), 15 g agar (Difco) l^{-1} distilled water, and 20 ml of antibiotic solution (which consisted of 250 mg ampicillin and 250 mg streptomycin sulphate (Sigma) 100 ml^{-1} distilled water). One isolated colony was transferred to 100 ml tryptone broth (10 g tryptone, 1 g dextrose, 5 g NaCl l^{-1} distilled water) containing 3 ml of antibiotic solution. The inoculated flask was incubated overnight at 37 °C without shaking to ensure pili retention. A 10 ml aliquot of the overnight *E. coli* culture (log_9) was transferred to 100 ml fresh tryptone broth to yield a final concentration of log_8 cfu ml^{-1} at 37 °C. Cells were incubated statically for 4 h. Logarithmic growth phase *E. coli* cells were used in the phage assays.

Phage enumeration

Direct assay. Samples with high phage levels were enumerated using the direct phage assay described previously by DeBartolomeis and Cabelli (1991).

Concentration assay. Centrifugation was performed according to DeBartolomeis and Cabelli (1991). The supernatant fluid was decanted into a sterile 250 ml polypropylene screw-top centrifuge bottle (Nalgene, Nalge Nunc International, Milwaukee, WI, USA) and tryptone and powdered beef extract (1 g each; Difco) were added. A 10 ml aliquot, per 150 ml of test sample, of the log phase F^+AMP *E. coli* culture was added to yield a final concentration of approximately 8 log cfu ml^{-1}. The mixture was incubated in a 35 °C water bath for 50 min, and manually inverted five times every 5 min of incubation. Afterwards, the mixture was centrifuged at 9000 *g* for 10 min at 4 °C. An 8 ml aliquot of the supernatant fluid was used to resuspend the pellet, which was transferred to two 5 ml double strength soft agar tubes (20 g Difco tryptone, 2 g Difco dextrose, 10 g Mallinkrodt NaCl and 14 g Difco agar l^{-1} distilled water). Tubes were gently hand shaken, then poured over a 150 × 15 mm tryptone agar plate containing antibiotic solution. After drying, plates were incubated for up to 18 h at 37 °C. Plaques were then counted with the aid of an illuminating box (Gagne Associates Inc., Binghamton, NY, USA). Positive and negative controls were performed for all phage samples. The positive control was 25 pfu ml^{-1} Group 1, F_2 phage solution (from W. Burkhardt). The negative control was sterile distilled water. Controls and experimental samples were processed identically. All phage samples were transported on ice to the Eastern Regional Research Center and processed within 1 h after sampling.

Bacterial enumeration

Test samples were assayed using Petri Film (3 M, Minneapolis, MN, USA) following the manufacturer's instructions. Testing included total aerobic plate count (TAPC), *E. coli* and total coliforms. Films were incubated at 37 °C for 48 h and quantified by manual counting. Samples were processed and the bacteriological determinations conducted at Hatfield Meats.

For spore analysis, 5 ml of scald tank water were heated to 75 °C for 20 min to kill all vegetative cells. Heated samples were then plated onto four brain heart infusion agar (BHIA) plates, and two plates were incubated aerobically at 37°C for 48 h. The other two plates were overlaid with Shahidi Ferguson Perfringens soft agar, then incubated anaerobically at 35 °C (Coy Instruments, Ann Arbor, MI, USA) for 48 h.

Waste treatment water

The water reclamation facility located at Hatfield Meats was previously described (Miller *et al.* 1994). It produces approximately 800 000 l d^{-1} treated water, containing 2–6 ppm chlorine, which is recycled for various unit operations within the plant, including the early stages of carcass dressing. Samples were obtained from seven sequential unit operations used to recondition all plant wastewater. Sampling sites included plant effluent, dissolved air flotation, denitrification, nitrification, clarification, pre-chlorination and chlorination. Water samples were collected either through a sampling valve (if present) or by dipping a sterile bucket into a pool of water. Water portions were transferred into sterile 250 ml polypropylene screw-top centrifuge bottles containing sodium thiosulphate (0·25 ml of a 10% sodium thiosulphate

solution) to remove any residual chlorine. The experiment was replicated 11 times from June to August 1994 in south-eastern Pennsylvania.

Hauling trailer floors

Fifteen hauling trailers were each evaluated for the efficacy of cleaning at six locations within the trailers, using a sampling protocol described by Rajkowski *et al.* (1998). Briefly, after animals were unloaded, bedding material at the sampling sites was removed by hand (covered with sterile gloves). A fresh glove was donned for microbiological sampling, which consisted of swabbing exposed floor surfaces, guided by the aid of a sterile stainless steel template with a 100 cm^2 opening. Swabs were taken at identical sites within each trailer cell floor location, as described previously (Rajkowski *et al.* 1998). Trailer floors were sampled using a sterile sponge (Whirl Pak®, Nasco, Fort Atkinsin, WI, USA) pre-moistened with 10 ml 1% buffered peptone water (BPW, Difco). Swabs were dragged perpendicularly (five motions per direction, 10 total). Exposed sponges were returned to the Whirl Pak bags. In the laboratory, 99 ml BPW were added to the Nasco bag and mixed for 60 s using a bench-top Stomacher 400 (Seward, London, UK). Samples were enumerated for bacteria and phage levels. After trailers were washed and sanitized, swab samples were collected as above, then diluted to double the volume (198 ml) using a 1% tryptone solution (to dilute any detergent or disinfectant effects on the *E. coli* lawn) for the coliphage assay.

Dressing operations water

Approximately 6000 market hogs (100 kg mean weight) were slaughtered each sampling day using dressing operations described previously (Miller *et al.* 1994). Reconditioned water was used at all unit operations up to, but not including, the pre-evisceration carcass washer. The pre-evisceration carcass washer, and all subsequent unit operations, employed potable water. Each operation contained its own water feed and drain. Water was collected using a 150 ml styrene foam cup on five different days from seven sites on the slaughter line, including the scald tank, de-hairer, gambrel table, polishers, pre-evisceration and final carcass washers. Water samples were collected and transferred to 250 ml polypropylene centrifuge bottles, then assayed as described above.

Scald tank water

Animals were scalded for 6 min by immersion in 60 °C water, containing no additives. Water overflow and replacement in the scald tank was 22·7 l min^{-1}. Water was collected approximately 30·5 cm below the water surface at seven sites along a squared, J-shaped scald tank. Sites one and seven were identified as the entrance and end of the scald tank, respectively. Sites two and three, and five and six, were chosen along the long and short arms of the tank, respectively. Site four was located at the bottom of the J-shaped tank. Water from each site was collected directly into a 100 ml Nasco bag and treated as above. Five replications were performed and enumerated for micro-organisms.

Carcass surfaces

Ten randomly chosen swine carcasses were sampled using sterile Nasco sponges re-hydrated with 10 ml 1% BPW. Sampling was performed twice, immediately after final carcass washing and 24 h after chilling. Using randomly chosen single sides from the split carcasses, sponges were applied, with the aid of a template, over a 100 cm^2 area of the jowl. Sponges were placed in a stomacher bag, diluted with BPW (40 ml), blended for 2 min using a Stomacher 400, then enumerated for micro-organisms.

Faecal microbiota

Colons from 20 freshly-slaughtered swine (10 carcasses, two trial days) were aseptically dissected and 25 g faeces were aseptically transferred to a stomacher bag containing 225 ml BPW. The faecal mixture was blended for 2 min using a Stomacher 400, then assayed and enumerated.

Data analysis

Data were analysed using SAS (V6·08, SAS Institute, Cary, NC, USA) general linear model or ANOVA techniques. When required, Bonferroni *t*-tests were used as a means separation technique (Miller 1981). The alpha level for significance for all tests was 0·05.

RESULTS

Waste water treatment effects

Population densities of the four groups of micro-organisms were assessed during seven water treatment stages. Initial levels (mean $\pm$ S.D.) of TAPC, phage, total coliforms and *E. coli* were 7·4 $\pm$ 0·4, 3·2 $\pm$ 0·5, 5·0 $\pm$ 0·5 and 4·7 $\pm$ 0·6 log cfu or pfu ml^{-1}, respectively. Fractional removal of phage and bacteria occurred as a result of the various treatment steps. However, the largest decreases occurred as a result of dissolved air filtration (stage 2), clarification (stage 5) and chlorination (stage 7). The TAPC levels were significantly lowered ($P < 0·05$) by 7·4 log cycles to undetectable as a result of the full process. Phage density decreased by 5·6 log cycles. Faecal bacterial indicators were lowered by a similar level. The coefficient of variation (CV) for all samples and

trials was largest for phage (372%), followed by *E. coli* (117%), total coliforms (91%) and TAPC (62%).

Efficacy of live haul trailer washing

Cleaning and sanitation significantly reduced ($P < 0{\cdot}05$) population densities of all micro-organisms on the floors of hauling trailers. Pooled data from the six hauling trailer floor locations from the 15 trailers are summarized in Table 1. After unloading, population densities on the floors were 6·8, −0·3, 4·6 and 4·3 log cfu or pfu cm^{-2}, for TAPC, phage, total coliforms and *E. coli*, respectively. Phage density was reduced by nearly 2 log cycles, TAPC levels were reduced by over 4 log cycles and *E. coli* and total coliforms were each reduced by over 3·5 log cycles, as a result of cleaning and sanitizing. No statistical differences were observed among the six sampling locations within each trailer ($P > 0{\cdot}05$), but highly significant differences ($P < 0{\cdot}01$) were observed among sampling dates. Variation among unwashed floor coliphage samples was high (CV = 330%) compared with the other indicator organisms (TAPC, 137%; total coliform and *E. coli*, 40%).

Dressing operations water

Microbiological water quality was evaluated at eight stages of the carcass dressing process. Unlike the wastewater treatment, which was continuous and sequential, the water used during carcass dressing was from a series of independent unit operations. In general, except for the scald tank (stage 1), water from the later stages of the dressing process contained fewer micro-organisms than from earlier stages. The scald tank water, which was maintained at 60 °C, exhibited the lowest mean bacterial population density of the various unit operation waters. Approximately 3 log cfu ml^{-1} TAPC, but no faecal bacteria, were detected. Based on this observation, it was hypothesized that surviving organisms included thermally-resistant aerobic spores. This was tested, and results are reported in the next section. Phage density in the scald water was $-0{\cdot}2 \pm 0{\cdot}0$ log pfu ml^{-1}. This was the third highest phage load among the eight unit operations, and probably reflected the thermal resistance of the virus. Among the waters from the eight unit operations, the highest TAPC was observed at the de-hairer (stage 2) and gambrel table (stage 3; 6 log cfu ml^{-1}). Phage were found in highest density ($1{\cdot}8 \pm 0{\cdot}6$ log pfu ml^{-1}) in de-hairer water. Total coliforms ($3{\cdot}5 \pm 0{\cdot}7$ log cfu ml^{-1}) and *E. coli* ($3{\cdot}3 \pm 0{\cdot}6$ log cfu ml^{-1}) levels were highest in the gambrel table water. This indicates that after scalding, the water, and probably the carcasses, become re-contaminated by de-hairing operations.

Population densities of all micro-organism groups were reduced throughout all dressing operations subsequent to the de-hairer or gambrel table. Excluding the scald tank, water indicator levels were lowest in the pre-evisceration carcass washer (stage 7; total coliform, $1{\cdot}2 \pm 0{\cdot}6$ log cfu ml^{-1}; *E. coli*, $0{\cdot}9 \pm 0{\cdot}7$ log cfu ml^{-1}) or after post-evisceration washing (stage 8; phage, $-1{\cdot}8 \pm 1{\cdot}1$ log pfu ml^{-1}; TAPC, $3{\cdot}1 \pm 0{\cdot}3$ log cfu ml^{-1}). Overall decrease in microbial populations in the eight process waters was largest for phage (3·6 log pfu ml^{-1}), followed by APC (2·9 log cfu ml^{-1}), *E. coli* (2·4 log cfu ml^{-1}), then total coliforms (2·4 log cfu ml^{-1}). For all water samples, phage density was lower than other micro-organisms, except in the scald tank.

Scald tank water

Despite the mixing that occurred by the movement of carcasses through the water in the scald tank, total coliforms were detected ($0{\cdot}2 \pm 0{\cdot}4$ log cfu ml^{-1}) only at the entry location (number one), suggesting that these vegetative cells entered with the animals and were quickly heat-killed. Phage density varied by only 1 log cycle throughout the process (range 0·3 to −0·4 log pfu ml^{-1}), indicating their thermal resistance. Total aerobic bacteria were found at all seven sampling points in the scald tank (range 2·7–3·1 log cfu ml^{-1}), confirming the observation reported in the previous section. Spore analysis indicated that aerobic spores (range 2·7–3·4 log cfu ml^{-1}) accounted for all of the TAPC. Further analysis

Table 1 Washing and sanitizing effects on the levels of micro-organism groups on floors of animal hauling trailers

Micro-organism group	After unloading animals	After sanitizing*
Phage	−0·33 (1·09)†	−2·15 (0·82)
Total aerobic plate count	6·81 (0·93)	2·48 (1·88)
Escherichia coli	4·30 (1·75)	0·53 (1·08)
Total coliforms	4·56 (1·81)	0·68 (1·20)

* Trailers were washed then sanitized using Roccal D™ (Rajkowski *et al.* 1998).
† Values are mean (S.D.) of log cfu or pfu per cm^2 from six pooled floor locations for 15 hauling trailers.
All row-wise reductions in indicator population densities were significant ($P < 0{\cdot}05$).

revealed an additional 3 log cfu ml^{-1} anaerobic spores at the seven sampling locations. Spores and TAPC were evenly distributed among the seven sampling locations.

Carcass surfaces

Microbial population densities on 10 freshly-dressed and chilled carcass surfaces were estimated using single-site (jowl) sponge samples (data not shown). Mean TAPC counts were 1·1 and 0·5 log cfu cm^{-2} for samples obtained before and after chilling, respectively. Standard deviations were 0·4 cfu cm^{-2}, regardless of sampling time. Total coliforms, *E. coli* and phage were not observed on the 10 carcasses.

Faeces

All 20 faecal samples contained phage (range 5·2–0·7 log pfu g^{-1}), with a mean density of $2·5 \pm 1·3$ log pfu g^{-1}. As expected, TAPC levels were highest ($8·5 \pm 0·5$ log cfu g^{-1}; min = 7·7, max = 9·2) among the micro-organisms tested. Total coliform and *E. coli* counts were $7·0 \pm 0·9$ (min = 5·6, max = 8·3) and $6·7 \pm 0·9$ (min = 5·5, max = 8·3) log cfu g^{-1}, respectively. The CV for phage density was 52%, approximately threefold higher than the faecal bacteria, and 10-fold higher than TAPC. An analysis of variance and Bonferroni *t*-test indicated a significant difference ($P < 0·05$) between the two trials for phage density values, but not for the other indicator micro-organisms.

DISCUSSION

The emergence or re-emergence of pathogenic micro-organisms (Vidaver 1996), interest in reclaiming and recycling water for food processing (Palumbo *et al.* 1997), concerns about the inadequacy of *E. coli*/coliform levels as indicators (Tyrrell *et al.* 1995; Rose *et al.* 1996) and its previous application to food systems (Kennedy *et al.* 1984, 1986a, 1986b), prompted this investigation of the potential of male-specific bacteriophage as a model organism. Bacteriophages have been proposed for an enteric pathogen index, an indicator system for enteric viral pathogens, and as a tool for studying mechanisms of viral disinfection (Maillard 1996). Within this broad class, male-specific bacteriophage was selected for application and evaluation in a pork slaughterhouse environment. The soft agar overlay plaque assay employed here proved to be easy to conduct and yielded overnight results that addressed the three objectives of this study.

The first objective of the study was to determine the occurrence of this organism in a pork slaughterhouse environment. Thus, a key result was the detection of phage in water effluent, animal hauling trailers and swine faeces from a major US pork packing-house. This confirms and expands on similar findings from Europe on the ecology of male-specific RNA coliphage (Havelaar *et al.* 1990). Phage densities in faeces in the present study (mean 2·5 log pfu g^{-1}), however, were lower by nearly 5 log cycles than those reported by Dutch investigators. The observation that phage was not found during a limited survey of pork carcasses may be due to assay insensitivity or to the fact that there was no faecal contamination present. The concomitant absence of total coliforms and *E. coli* supports the latter theory. A more extensive study should be conducted to determine the general incidence and density of coliphage on swine carcasses.

The high variability of phage densities in wastewater (372% CV), on unloaded trailer floors (330%) and in swine faeces (52%) suggests sporadic occurrence and levels in swine faeces. Havelaar *et al.* (1990) identified coliphage in swine faeces but observed F+-specific RNA bacteriophages in human and animal faeces inconsistently. Thus, the high variability and inconsistent association of phage with faeces may decrease its usefulness as a contamination indicator organism.

A second objective of the study was to evaluate phage as a process integrity indicator. In general, we found that where sequential process steps were employed, virus densities were clearly and significantly decreased. This suggests that male-specific bacteriophage may serve as a promising microbial indicator for the pork slaughter environment. Support comes from the wastewater treatment data, which demonstrated a 5·6 log pfu ml^{-1} coliphage reduction; this is similar to that achieved for traditional faecal indicators. Also, the trailer washing experiments showed that cleaning reduced levels of all micro-organisms. These results support and expand those of Rajkowski *et al.* (1998) who demonstrated that washing and sanitizing swine hauling trailers significantly decreased the incidence of *Salmonella* and *E. coli*. Furthermore, the evaluation of carcass dressing unit operations revealed that phage densities followed similar reduction patterns to the other micro-organisms. The greater thermal resistance of the phage may be of value as an indicator of the efficacy of the scald tank in minimizing microbial populations on carcasses. Spore density may also fulfil this role. The present research results, however, suggest that male-specific bacteriophage may be most useful for verifying the integrity of wastewater treatment.

The final study objective was to compare male-specific bacteriophage with traditional micro-organisms associated with faecal contamination. Regression analysis of virus densities *vs* levels of the other microbial indicators was conducted across all study samples (> 400). Analysis of wastewater treatment samples yielded a correlation coefficient (r^2) of 0·85 ($P < 0·05$) between phage and *E. coli* population densities, and $r^2 = 0·83$ ($P < 0·05$) with total coliform levels. For trailer floor swabs, significant correlations ($P < 0·05$) of 0·60 and 0·61 were determined between phage and coliform, and *E. coli*, respectively. While these correlations were high, correlations from other parts of the study were not significant

($P > 0{\cdot}05$). For example, an overall r^2 value of $<0{\cdot}05$ was estimated between phage and total coliform or *E. coli*. This may be partly subject to bias because samples included scald tank data which contained very few thermally-sensitive coliforms and no *E. coli*. Yet, regression analysis of the swine faecal data yielded correlation coefficients of 0·03 and 0·04 between coliphage and total coliform and *E. coli*, respectively. Donnison and Ross (1995) studied coliphage and bacterial indicators in waste treatment lagoons, including those containing animal processing waste. These investigators similarly found a low correlation between male-specific bacteriophage and *E. coli* population densities.

Although not studied in the present research, other investigators have attempted to use phage as a pathogen index. For example, Martinez-Manzanares *et al.* (1991) concluded that the presence of coliphage in shellfish is valid for establishing the potential health hazard associated with the presence of *Salmonella, Vibrio parahaemolyticus* and *Staphylococcus aureus*. However, Vaughn and Metcalf (1975) failed to correlate coliphage and enteric virus densities in estuarine waters and oysters. Moreover, Carducci *et al.* (1995) reported that coliphage and enterovirus densities were not related in sewage and aerosol from activated sludge. Despite these shortcomings, bacteriophage may prove to be a better index than traditional organisms such as total coliforms and *E. coli*, as these bacterial groups are inadequate for ensuring safety from emerging protozoan parasites including *Cryptosporidium, Giardia* and *Cyclospora*. Therefore, further studies need to be conducted in order to determine whether bacterial viruses are suitable index organisms for a variety of microbial pathogens.

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